THE ANTI-NEURITIC BASES OF VEGETABLE ORIGIN IN RELATIONSHIP TO BERI-BERI, WITH A METHOD OF ISOLATION OF TORULIN, THE ANTI-NEURITIC BASE OF YEAST

BY

E. S. EDIE, W. H. EVANS, B. MOORE, G. C. SIMPSON, AND A. WEBSTER

From the Laboratories of Tropical Medicine and Bio-Chemistry of the University of Liverpool

(*Received for publication* 29 *May*, 1912)

In a recent paper[1] from these laboratories an account was given of the chief investigations into the causation of Beri-beri up to 1910. This account was largely an abstract of the masterly monograph in which Schaumann[2] described the work of other investigators, added much important work of his own, and brought evidence to suggest that other diseases might have a similar etiology to Beri-beri.

Some of our earlier results were briefly mentioned in that paper, and the account of our further researches will be prefaced by a brief summary of some of the numerous papers on this subject which have appeared in the interval.

One of the most interesting papers gives an account of the later researches of Fraser and Stanton[3] who have already contributed so largely to the knowledge of this subject.

They demonstrate that the active anti-neuritic substance of rice meal is soluble in water and in alcohol, show that it is stable in acid, unstable in alkaline solution (p. 74), and that its thermolability varies with varying physical factors. They show that it is not a phytinbody or a fat, and that it probably is not a protein nor does it contain phosphorus.

They further confirm the fact that the phosphorus content of a rice is an indicator of its safety as an article of diet, and, with a view to the prevention of Beri-beri, recommend administrative measures to prevent the sale in the Malay States of rice with less than 0·4 % phosphorus pentoxide.

Eijkman[1] recapitulates his earlier work on the subject, and all must regret that he and his colleagues were prevented from continuing their work in Batavia and Java *where, in the years* 1889 *to* 1897, *they had so far advanced the study of Beri-beri and Polyneuritis on experimental lines, and had demonstrated that Beri-beri could be cured and its occurrence prevented by the use of hand-milled rice.*

His further researches to isolate the active substance from rice meal are recorded. Cure of polyneuritis in fowls was effected by two or three doses of extracts of rice meal; less than five grams of extract containing 0·085 % P_2O_5 and 0·012 % N, sufficed to restore to activity birds severely lamed. He strongly opposes Schaumann's theory (1910) that the active substance is a phosphorus-containing compound, though awaiting fuller accounts of his later communications[5].

Shiga and Kusama[6] report extensive investigations disproving the bacterial and toxic theories of Beri-beri, and confirm much of Eijkman's, and Fraser and Stanton's work.

Kilbourne[7] shows that the potassium content of rice meal is of almost equal value to the phosphorus content as an indicator of its safety.

Highet[8] in Siam, and Aron[9] and Heiser[10] in the Philippines, have shown that Beri-beri followed the introduction into those countries of steam milled rice, and that the disease disappeared from institutions (jails, etc.) on the re-introduction of hand-milled rice. They have further demonstrated that rice can be milled by machine, without impairment of keeping powers and palatability, and with the retention of the valuable outer layers, which render it free from the risk of inducing Beri-beri.

Their work is most interesting and important from the administrative point of view, and has already led to the diminution of Beri-beri in their districts. A Bill was introduced into the Philippine Legislature to discourage the use of polished rice, and

prevent Beri-beri, by putting a duty on rice with a phosphorus content less than 0·4 % P_2O_5—the same limit that Fraser and Stanton have suggested in the Malay States. Such rice is already prohibited in Government Institutions by administrative orders.

Many of these papers were read at a Meeting of the Far Eastern Association of Tropical Medicine (Philippines, 1910), and a resolution was adopted affirming strongly that Beri-beri is a disease due to the continuous consumption of white polished rice.

Chamberlain and Vedder[11] following Fraser and Stanton, showed that an extract of rice meal in 70 % alcohol, concentrated at a low temperature till alcohol free, maintains its activity to cure polyneuritis. They further showed that the active substance is able to dialyze through parchment. A daily dose of these extracts, containing 0·16 mg. P_2O_5 and 4·06 mg. Nitrogen, cured, in a few days, fowls severely lamed.

In another contribution[12] they confirm these results, having kept fowls for 100 days on polished rice and the extract without development of neuritis. They find that the sucrose and ash in the extract are inactive, and so exclude 0·91 of the 1·34 % solids in the extract.

In addition, they find that the active substance is absorbed by bone-black, and are now proceeding to attempt to isolate and analyse it.

Funk[13] has isolated from rice meal a crystalline nitrate of an organic base which is extremely active in reviving pigeons with polyneuritis from feeding on polished rice.

The necessary dose contains about 4 mgs. of Nitrogen, corresponding to 0·05 gram. of the nitrate of the base, to which he allots the provisional formula $C_{17}H_{18}O_4N(HNO_3)$.

The crystals were in the form of microscopic needles, melting at 233° C., insoluble in cold water or alcohol, soluble, with difficulty, in hot water. They were free from ash and from chlorine and sulphuric acid.

This is the first record of the isolation and analysis of an active substance, and the method by which it was obtained will be briefly described. One and a half kilograms of rice meal was extracted with four litres of acid alcohol; separation of the filtrate was completed by the hydraulic press; about three and a half litres of extract were obtained, and evaporated in vacuo at 30°, leaving a fat-like residue. This was melted and treated with water, and filtered while warm. The aqueous part was treated with ether to remove all fatty substances; it cured pigeons in doses corresponding to 20 grams of the original polishings.

The total aqueous extracts from 54 kilos rice meal amounted to 17 litres, which was treated with sulphuric acid and phosphotungstic acid throwing down 900 grams of precipitate. The precipitate was dried, washed with 5 per cent. H_2SO_4, ground with baryta and shaken three hours with water. The precipitate was filtered off, the filtrate smelt of ammonia and methylamine. The baryta was precipitated with sulphuric acid, and the filtrate neutralised with hydrochloric acid, and evaporated in vacuo at room temperature. The residue was extracted with alcohol, and the alcoholic solution was active in doses = 40 grams of rice polishings. The solution was free from proteins, phosphorus, and carbohydrates.

The alcoholic solution gave a crystalline precipitate with mercuric chloride, which was separated, washed, and recrystallised from water; this consisted mostly of cholin, but some active substance was also present. Active substance was present in both the alcoholic and aqueous filtrates.

Aqueous filtrate. The mercury was removed, and the filtrate evaporated and taken up in alcohol was treated with platinic chloride to remove cholin. After removing the platinum from the filtrate it was treated with phosphotungstic acid, giving a crystalline precipitate which yielded an active substance when freed from phosphotungstate with baryta and carbon dioxide.

Alcoholic filtrate evaporated and dissolved in water: mercury removed by sulphuretted hydrogen; chlorine, &c., were removed by successive treatment with silver sulphate, sulphuretted hydrogen, and baryta. The alkaline solution was precipitated with silver nitrate and baryta, the precipitate decomposed with sulphuretted hydrogen and freed from silver and barium. It proved active, and, after evaporation in vacuo, crystals were with difficulty obtained from alcohol, with the composition, &c., given above.

ACTIVITY OF VARIOUS FOOD STUFFS

We have continued the attempt, by the use of various additions to rice diet, to prevent or delay the onset of polyneuritis or to cure animals already suffering from it.

In the earlier experiments phytin, nucleins, caseins, lecithins, etc., were used, in part from commercial sources and in part isolated by us (using care to avoid the use of strong reagents).

Addition of commercial nuclein and lecithin to the diet of polished rice had no apparent influence either on the time of onset of incapacity or death or on the loss of weight. Addition of casein seemed to postpone for a few days (five) the onset of incapacity and death, and the birds lost on the average considerably less weight. A mixture of casein, nuclein and lecithin also proved an ineffective addition.

The addition of an acid extract of rice meal, neutralised by bicarbonate of soda and taken to dryness on a water bath, led to a very rapid loss of weight; in the light of further results this extract would appear to have been 'denaturised' by excess of alkali or overheating.

Phytin extracted from rice meal, but not re-purified, prolonged the life of the birds distinctly, and lessened the wasting to a fair extent: this may have been due to the adhesion of a small amount of the active material to the phytin.

Casein, separated from milk and not re-purified, again seemed to postpone the onset of lameness and to lessen the loss of weight. All four birds were alive after thirty days on this diet—one decidedly lame, two slightly lame, the other not affected. With non-protective substances some birds would certainly have died earlier than this.

Lecithin was prepared from egg yolk: part was separated from solution by cooling apart by acetone precipitation. This also proved inactive.

In these experiments casein and phytin alone seemed to contain any of the active principle. We also thought that certain sapoglucosides (saponin and hederin) had had a slightly favourable influence, but the experiments had to be abandoned owing to severe diarrhoea at an early period.

BIO-CHEMISTRY OF EXTRACTS OF RICE MEAL AND YEAST

Among other points, we find that nearly twice as much of the phosphorus of rice meal goes into solution in water after denaturisation at 120° C. Of the soluble phosphorus of rice meal nearly five-sixths dialyses: of the soluble phosphorus of denaturised rice meal only two-thirds dialyses. The protective properties of the fractions separated were not tried, as the quantities were insufficient for continued feeding experiments.

More than twice as much of the phosphorus of dried yeast, after denaturisation at 120° C., appeared as 'phosphatide phosphorus.'

Alcoholic (90 per cent.) extract of rice meal was found to be active (as stated by Fraser and Stanton) in protecting birds from the onset of neuritis, and in curing them, but concentration on the water bath rendered the extracts inactive.

The extracts were concentrated under a fan at room temperature to small bulk till all smell of alcohol had disappeared. These extracts preserved some activity (cp. Chamberlain and Vedder, loc. cit.). Four birds, which were very weak and disabled with polyneuritis, were each given the extract from 25 grams of rice meal, daily: in the first week they showed a decided improvement; became more active; three gained 5 %, 15 % and 8 % in weight, respectively, in one week (loss previous to treatment, 32 %, 30 % and 36 %). The other, though it became more active at first, lost

a further 2 % in seven days, and 14 % in ten days, being then 47 % below its original weight. It died on the 15th day of feeding, 7 % further loss, but for the last week had been receiving small doses of Hederin daily.

Of the other three birds, two fell in weight (5 % and 2 %) between the seventh and tenth days, the other one gained a further 2 %; two fell again in weight, one losing a further 5 %, the other falling 5 % more to nearly its weight at the commencement of treatment. The last, on Hederin, just maintained its increase. All were now extremely weak again, and could scarcely survive more than a day. They were now put on yeast, and rapidly improved, walking and flying well in a few days, and gaining, respectively, 9·6 and 6 % in the week.

Attempts were made to precipitate the active principle from these extracts by the lead acetate method. Neither the normal or basic lead acetate precipitates proved active: the filtrate, however, was active: the precipitate from this by phosphotungstic acid did not, however, prove active.

Funk's method was now tried direct on the original extract of the meal. *A strong odour of ammonia and methylamine was noticed in the treatment with baryta.* In spite of continued treatment with this extract, the birds died in a few days. (? Cholin poisoning, C. & F.).

We considered that other foodstuffs might give more favourable results, and so, for the present, abandoned the investigation of rice meal.

On account of the resemblance of the active substance in solubility, etc., to some of the peculiar lecithins and bases described by Winterstein in wheat meal, we tried the lead acetate precipitable portions of Katjang beans, but found them inactive. Feeding with fresh brain also failed to preserve birds from death when incapacitated with neuritis.

An attempt to isolate the active substance from Katjang beans proved unsuccessful, neither the lead acetate filtrate nor precipitate proving active. Further experiments will be made.

Natural yeast had been previously found to possess marked preventive and curative properties, and extracts from yeast were next investigated.

INVESTIGATION OF YEAST EXTRACTS

After various experiments[16] the following method was adopted: Twenty pounds of commercial fresh pressed yeast were extracted in the cold with successive quantities of methylated spirit, using in all about twenty litres of spirit; the yeast was filtered through thick calico, and the alcoholic filtrate was freed from alcohol at room temperature by means of an electric fan. There remained about seven litres of watery fluid, dark yellow in colour, smelling strongly of beer, and with an intense bitter taste.

This water extract was mixed with sufficient plaster of Paris to make it 'set.' The plaster matrix, after standing overnight, was ground to a fine powder, and extracted in the shaking machine with successive small quantities of methylated spirit made faintly acid with hydrochloric acid. These extracts were freed from alcohol, as before, and the watery fluid obtained, amounting to 3-4 litres, was precipitated with excess of basic lead acetate. The lead precipitate, having previously been found to be inactive, was discarded. The filtrate was freed from lead with sulphuretted hydrogen, and then concentrated to a syrup in vacuo at 38° C. This syrup was treated with absolute alcohol, and the sticky hygroscopic yellow precipitate (creatinin, etc.) was filtered off. The alcoholic filtrate was again freed from alcohol and then precipitated with baryta and silver nitrate. This precipitate was decomposed with sulphuretted hydrogen, filtered, excess of sulphuretted hydrogen removed by the fan, and then taken to dryness in vacuo at 38° C. A small quantity of a brown, sticky, hygroscopic mass was obtained in this way, easily soluble in cold water, and intensely active.

A dose of 0·006 gram administered to a bird with severe convulsions and lameness, improved the convulsions in four hours: the bird was flying strongly in twenty hours, and the lameness disappeared in forty-eight hours. Two further doses of 0·003 gram were given on the third and eighth day; the bird appeared normal, and gained weight on polished rice diet, but died on the fifteenth day without return of lameness or convulsions. Similar results were obtained with other birds, but there was not sufficient substance to make prolonged experiments to see if the substance would restore the birds to their original weight and condition, etc.

The substance was further purified by treatment with alcohol: it was insoluble in ether and acetone, and on standing yielded feathery crystals identical with those found in Experiment X.[16]

The ash consisted principally of barium nitrate and a small amount of phosphate, which may not prove to be merely impurity.

Pending further investigations into the exact nature of the ash and its relationship to the organic compound, it is assumed to consist entirely of impurity, and the composition of the residue is approximately—

C = 40·5
H = 8·07
N = 13·32
O = 38·11

100·00

This corresponds to the formula $C_7H_{17}N_2O_5$ or $C_7H_{16}NO_2(HNO_3)$.

As the action of baryta splits off trimethylamine we may assume the presence further of this group, and write it:—

$$N(CH_3)_3 \ . \ C_4H_7O_2 \ . \ (HNO_3).$$

The substance isolated by us from yeast gives an oily precipitate with gold chloride (resembling Kutscher's bases), but we have not been able to obtain, as yet, enough of the gold salt for identification.

Attempts are being made to isolate larger amounts of the active principle, and to work out more completely its constitution and its influence on metabolism. It is also important to determine if the antineuritic bases of rice, wheat, and other foodstuffs are the same as that of yeast or different (as Funk's results indicate), and also whether they are breakdown products of more complex substances present in the foodstuffs.

Part of the funds for this research were allotted by the Advisory Committee for the Tropical Diseases Research Fund (Colonial Office) to the Liverpool School of Tropical Medicine.

REFERENCES

1. Simpson and Edie. Annals of Tropical Medicine, Vol. V, 1911.
2. Schaumann. Archiv. f. Schiffs- und Tropen-Hygiene, Vol. XIV, Beiheft 8, 1910.
3. Fraser and Stanton. 'The Etiology of Beri-Beri,' Studies from Institute for Medical Research, Malay States, No. 12, also Philippine Journ. of Science, Vol. V, p. 55, 1910.
4. Eijkman. 'Polyneuritis Gallinarum and Beri-beri,' Archiv. f. Schiffs- und Tropen-Hygiene, Bd. XV, p. 698, 1911.
5. Schaumann. Transactions of Society of Tropical Medicine, 1911.
6. Shiga and Kusama. Archiv. f. Schiffs- und Tropen-Hygiene, Vol. XV, 1911.
7. Kilbourne. Philippine Journal of Science, Vol. V, p. 127, 1910.
8. Highet. Philippine Journal of Science, Vol. V, p. 73, 1910.
9. Aron. Philippine Journal of Science, Vol. V, pp. 81 and 98, 1910.
10. Heiser. Philippine Journal of Science, Vol. VI, p. 229, 1911.
11. Chamberlain and Vedder. Philippine Journal of Science, Vol. VI, p. 251, 1911.
12. Chamberlain and Vedder. Philippine Journal of Science, Vol. VI, p. 395, 1911.
13. Cooper and Funk. Lancet, No. 4601, 1911.
14. Funk. Journal of Physiology, XLIII, p. 395, 1911.
15. Kutscher. Zeitschrift f. physiol. Chem., Bd. LI, 1907.
16. Edie, Evans, Moore, etc. Bio-Chem. Jour., Vol. VI, p. 234, 1912.